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Epristeride is a Selective and Specific Uncompetitive Inhibitor of Human Steroid 5α-Reductase Isoform 2

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Specificity of an enzyme inhibitor can have profound implications upon the compound's therapeutic potential, utility and safety profile. As potent inhibitors of human steroid 5α-reductase (SR) the 3-androstene-3-carboxylic acids, or steroidal acrylates, may be useful in treatment of diseases such as benign prostatic hyperplasia for which 5α-dihydrotestosterone (DHT) appears to be a causative agent. To determine its specificity profile, the interactions of a representative compound from this class, *N*-(*t*-butyl)androst-3,5-diene-17β-carboxamide-3-carboxylic acid (epristeride, SK&F 105657), have been studied with 7 other steroid processing enzymes and 5 steroid hormone receptors. The affinity of epristeride for each of these 12 potential targets was found to be at least 1000-fold weaker than that for SR, the intended target. In addition, using samples of the individually expressed two known forms of human SRs, epristeride has been shown to be a selective inhibitor of the recombinant human SR type 2, the predominant activity found in the prostate of man. Nonetheless, the mechanisms of SR inhibition for both isoenzymes involve formation of a ternary complex with epristeride, $NADP^+$, and enzyme. Epristeride, consequently, has been shown to be an uncompetitive inhibitor versus steroid substrate of both human SR isoenzymes. These results suggest that this 3-androstene-3-carboxylic acid is a specific and selective inhibitor of the human type 2 SR, and that epristeride is an attractive compound for further investigation as a safe and effective therapeutic agent in the potential treatment of disease states associated with DHT-induced tissue growth.

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INTRODUCTION

Benign prostatic hyperplasia (BPH) is an age-related, progressive disease that afflicts a high percentage of men over 50 years of age. Due to the circumjacent relationship of the prostate to the urethra, glandular enlargement can result in compromised urinary function requiring medical treatment. Historically, therapy most often has involved surgical procedures [1]. During the last few years, advances have been made in alternative approaches toward intervention of the underlying factors contributing to prostatic growth and progression of the disease such as selective hormonal manipulation.

It is well established that growth of the prostate is stimulated by androgens; more specifically, 5α-dihydrotestosterone (DHT) appears to bear primary responsibility for the trophic support of this organ [2, 3]. Preclinical studies focussing on steroid 5α-reductase (SR), the enzyme that converts the classical androgen testosterone (T) to DHT, have demonstrated selective retardation of prostatic growth coincident with suppression of DHT biosynthesis [4, 5]. Consequently, selective inhibition of SR could offer an alternative treatment for diseases such as BPH where tissue growth is DHT responsive. Such specific androgen ablation would be expected to maintain T supported functions, avoiding potential undesirable effects associated with total androgen withdrawal.

As a result of our research efforts utilizing strategies of rational inhibitor design, the 3-androstene-3-carboxylic acids or steroidal acrylates have been identified as a new class of SR inhibitor [6]. Potent inhibition of enzyme activity with these and structurally related compounds has been demonstrated with SR activity

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from human prostate [7, 8], rat prostate and liver [9], and monkey (*Cynomolgus*) prostate [10]. These compounds, exemplified by *N*-(*t*-butyl)androst-3,5-diene-17β-carboxamide-3-carboxylic acid (epristeride, SK&F 105657, compound 1), have been shown to interrupt SR catalysis by formation of a tightly associated ternary-complex with enzyme and $NADP^+$ on the product side of the ordered kinetic profile [9]. A kinetic consequence of this mechanism is the relatively uncommon classification of the steroidal acrylates as uncompetitive inhibitors [6–9].

Recently, two distinct human proteins which can catalyze the SR reaction have been identified [11–14]. Comparison of the predicted primary amino acid sequences for the two isoenzymes shows only 50% homology, suggesting differing forms, functions, and/or regulation. The catalytic properties of the two human SR enzymes have been shown to differ in several characteristics following cloning and expression [11–14]. For example, isozyme 1 shows maximal catalytic velocity over a pH range of 6.5 to 8.5 while isozyme 2 has a narrow maximal range at acidic pH (4.5–5.5) [13, 14]. In addition, the two isozymes have been reported to respond differently to MK-906 (finasteride), a competitive inhibitor of human prostatic SR currently available for treatment of BPH [13, 14]. The characteristics associated with isozyme 2 are most similar to the predominant SR activity detected in human prostate tissues, although mRNA for both proteins have been detected in human prostate [13, 14].

Potential therapeutic agents based on considerations of the mechanism of the enzyme target and which inhibit by unique chemical and kinetic mechanisms should impart a therapeutic advantage through a well-defined specificity of action. Yet a lack of target selectivity could compromise potential therapeutic utility in man. Consequently, discovering whether members of the steroidal acrylate class of inhibitors would demonstrate any unwarranted effects on alternative enzyme or receptor systems was considered important prior to any clinical evaluation. Based on its potent inhibition of SR activity derived directly from human prostatic tissue [7], epristeride (compound 1) was selected from this class of uncompetitive inhibitors for further evaluation and characterization. In this manuscript we describe the results from studies that demonstrate the specificity of epristeride as an inhibitor of SR versus that of several enzymes having features of ligand binding and chemical catalysis common to that for SR. In addition, the effects of epristeride upon ligand binding to five steroid-hormone receptors have been studied. The selectivity of SR isoenzyme inhibition by epristeride was also investigated using independently expressed samples of the two recombinant human proteins.

EXPERIMENTAL

Materials

[4-^{14}C]T (55–57 mCi/mmol) was purchased from Amersham Corp. (Arlington Heights, IL) and New England Nuclear (NEN, Boston, MA). [1,2-^{3}H]T (49 Ci/mmol), [7-^{3}H]dehydroepiandrosterone sulfate ammonium salt (16.3 Ci/mmol), [4-^{14}C]pregnenolone (57.2 mCi/mmol), [9,11-^{3}H]androsterone (53.3 Ci/mmol), Econosolve II, and Aquasol 2 were obtained from NEN. Epristeride (SK&F 105657) was prepared as described previously [7, 8]. Radioactivity was determined with a Beckman LS-5801 scintillation counter calibrated to disintegrations per min (dpm) with Beckman standards or with a System 2000 Bioscan Imaging Scanner (Bioscan, Washington, DC). Single wavelength UV/vis spectral changes were monitored on a Gilford 260 spectrophotometer. Analyses of enzyme reactions were performed on prechanneled silica TLC plates containing a preabsorbing region (Si250F-PA, Baker) or plastic backed TLC plates (Kieselgel 60 F_{254}, Merck, Germany).

Cloning of SR enzymes

Total RNA was prepared from human prostate tissue by the guanidinium isothiocyanate method [15] and poly $(A)^+$ RNA was isolated using a mRNA purification kit (catalog No. 1353616) supplied by Boehringer Mannheim Corp. (Indianapolis, IN). A custom cDNA library cloned into lambda ZAP II arms was prepared by Stratagene Cloning Systems (La Jolla, CA). In order to prepare the human SR type 1 for optimal expression in mammalian cells as discussed [12], oligonucleotide primers based on its reported DNA sequence [12] were prepared and used to obtain a 636 base pair (bp) DNA fragment from 1 ng of clone ph5α 45 (provided by Dr David Russell) using the polymerase chain reaction (PCR) [16]. This DNA fragment, which encompassed DNA sequences between nucleotide positions 234 and 870 of the human SR type 1 cDNA, was ^{32}P-labeled and used to probe the human prostate cDNA library by *in situ* plaque hybridization as described previously [17]. Eight positive clones were plaque purified and the cDNAs were excised to yield recombinant Bluescript plasmids in *E. coli* XL1-Blue cells according to the method recommended by Stratagene. Recombinants were analyzed by DNA sequencing using a Sequenase kit (U.S. Biochemical Corp., Cleveland, OH). Four clones were identified that contained full-length cDNAs identical to the reported human SR type 2 cDNA [13].

Plasmid construction and Chinese hamster ovary (CHO) cell expression

In order to prepare the SR type 1 cDNA for expression in CHO cells, a 132 bp DNA fragment was synthesized that contained from its 5′ to 3′ termini the following: an *Eco* RI site, a consensus Kosak sequence for optimal expression [18], an ATG initiation codon, and 115 bp of amino terminal coding sequence of the human SR type 1 protein ending in an *Eag* I site. This fragment, combined with an 698 bp *Eag* I–*Hind* III fragment that harbored the remaining coding region of the SR type 1 protein, a TAA translation stop codon, and 37 bp of 3′ untranslated region, was ligated into the

Eco RI–*Hind* III site of the CHO expression vector pCDN. The resulting vector, pCDNhSRty1, contained the entire SR type 1 cDNA within an eucaryotic expression cassette. For the expression of the human SR type 2 gene, oligonucleotide primers were synthesized corresponding to the amino and carboxy termini of the SR type 2 protein and used to obtain an 806 bp DNA fragment from template full-length SR type 2 cDNA using PCR technology. The PCR primers included *Eco* RI and *Hind* III restriction sites at their 5′ and 3′ ends, respectively, to facilitate subcloning into those sites within the pCDN vector. The resulting vector, pCDNhSRty2, contained the entire SR type 2 cDNA within the pCDN vector expression cassette. The DNA sequence fidelity of the cDNA inserts of the pCDNhSRty1 and pCDNhSRty2 clones were confirmed by complete DNA sequence analysis. To produce stable CHO cell transformants, pCDNhSRty1 or pCDNhSRty2 DNA were linearized and independently introduced into CHO cells by electroporation. Populations of stable transformants expressing SR enzymatic activities were selected using G-418 media as described previously [19].

SR

SR assays were conducted essentially as described previously [7, 9, 20, 21]. Assays of enzyme from rat prostate and liver were conducted at pH 7.0 (20 mM sodium phosphate) while those derived from human prostate and rat epididymis were conducted in 50 mM sodium citrate at pH 5.0. The recombinant human SR type 1 was evaluated in 20 mM sodium phosphate, pH 7.5 while 50 mM sodium citrate, pH 5.0, was used for assays of recombinant human SR type 2. All enzyme preparations were microsomal or particulate fractions of cell or tissue homogenates, where the SR activites were demonstrated to be localized.

3β-Hydroxy-5-ene-steroid-dehydrogenase/3-keto-5-ene-steroid isomerase

The coupled enzyme activities of 3β-hydroxy-5-ene-steroid dehydrogenase and 3-keto-5-ene-steroid isomerase were derived from bovine adrenals as described previously [22]; enzyme activities were monitored by following the conversion of [^{14}C]pregnenolone to progesterone as we have outlined [22]. Inhibition studies were conducted in 100 mM phosphate buffer, pH 7.5 in the presence of 1 μM pregnenolone and 125 μM NAD^+.

3β-Hydroxysteroid dehydrogenase/30α-decarboxylase

The last steps of the enzyme catalyzed removal of the C-4 methyl groups of lanosterol were evaluated with a rat liver microsomal solution prepared by the procedure described by Brady and Crowder [23]. Enzyme activity was monitored with [30-^{14}C]cholestanol-4α-carboxylic acid (35.5 μCi/μmol) as substrate which was specifically prepared for use in this evaluation [24]. Incubations were conducted at 37°C in 1 ml of 100 mM sodium phosphate, pH 7.4, containing 4 mg/ml Tyloxopol with 100 μM NAD^+ using septum sealed vials containing a CO_2 well-trap (Pierce Chemical Company, Rockford, IL). Upon stopping the reactions by injection of 100 μl of 6 N H_2SO_4, the released [^{14}C]CO_2 was trapped in a solution of 0.5 M hydroxylamine in 10% KOH (100 μl) added to the well-trap by syringe.

Following vigorous agitation for at least 1 hour to effect equilibrium, the well and its contents were removed from the vial, added to 15 ml Aquasol II, and the radiochemical content determined as a measure of enzyme activity. Using this procedure, values of K_m for cholestanol-4α-carboxylic acid and NAD^+ were estimated to be 10 μM and 90 μM, respectively [24], upon fitting initial velocity data to the SEQUEN program [25]. Inhibition studies were conducted at 1.9 μM [30-^{14}C]cholestanol-4α-carboxylic acid (68400 dpm, 0.03 μCi) with 100 μM NAD^+.

Steroid 5β-reductase

The soluble steroid 5β-reductase from rat liver was partially purified according to the procedure of Okuda and Okuda [26] through the DEAE–Sepharose CL-6B step. The active fractions from this column were pooled and stored at 4°C; minimal loss of activity was observed after 30 days at 4°C. The assays for 5β-reductase involved measuring conversion of [^{14}C]T to 5β-dihydrotestosterone plus its metabolite 5β-androstane-3α,17β-diol. As with the previously published assay for SR [9], steroidal substrates and inhibitors in ethanol were deposited in test tubes and the solvent was removed. Enzyme activity was evaluated over a period of 20- to 50-min incubations at 30°C containing 400 μM NADPH and 2 μM [^{14}C]T in 100 mM Tris buffer (pH 7.4) at a volume of 0.5 or 1.0 ml. The reaction was quenched as described for SR [9]; separation of substrate and products was accomplished by TLC, twice eluting with cyclohexane–ethyl acetate (1:1, v/v). The relative amount of radiolabel in the substrates and products within each assay was determined on a Bioscan Imaging Scanner, from which enzyme activity was determined as for SR [9].

3α-Hydroxysteroid dehydrogenase

Rat liver 3α-hydroxysteroid dehydrogenase was purified through the ammonium sulfate precipitation as described by Penning *et al.* [27]. The solution of the suspended pellet from the 40 to 75% ammonium sulfate fraction was dialyzed three times against 10 mM Tris, pH 8.6, with 1 mM EDTA and 1 mM dithiothreitol, and a fourth time against the same buffer containing 20% glycerol. The enzyme was stored at −80°C. Enzyme activity was monitored by following the conversion of [^{3}H]5α-androsten-3α-ol-17-one to 5α-androstane-3,17-dione. Incubations contained 200 μM $NADP^+$, 1.9 μM [^{3}H]androsterone (0.1 μCi) in 100 mM potassium phosphate buffer (pH 7.0) in a final volume of 1.0 ml. Assays were initiated by addition of the enzyme, diluted in 10 mM phosphate buffer, pH 7.0, containing 10 mg/ml bovine serum albumin, to give maximal turnover of substrate of 20%. Reactions

were incubated at 30°C for 10 to 20 min, and quenched with 4 ml ethyl acetate. The separation of substrate (androsterone) and product (androstanedione) was effected by TLC (once) in chloroform–acetone (9:1, v/v) [9].

Aromatase

The aromatase activity in the microsomes of human term placenta was assayed essentially as described previously [28, 29]. All steroids, substrates, and inhibitors in ethanol were deposited in the reaction tubes and the solvent was removed under a stream of dry argon before introduction of the other reaction components. Potential inhibition of aromatase activity was determined at 20.6 nM [1,^{3}H]androstenedione (0.02 μCi/assay) with 400 μM NADPH in 0.1 M sodium phosphate buffer at pH 7.6 in a final volume of 1.0 ml. Incubations were conducted at 37°C for 10 min, and enzyme activity determined as published [29].

Steroid sulfatase

Rat liver microsomes, prepared as described by Nilsson *et al.* [30] were used as the source for steroid sulfatase activity. Activity was determined with [7-^{3}H]dehydroepiandrosterone sulfate as published [30]. All assays were conducted in 0.5 ml of 100 mM sodium phosphate, pH 7.0, buffer at 37°C. Incubation times typically were 30 min with hydrolysis product separated from substrate by extraction into toluene. Inhibition studies included 10 μM (0.2 μCi) of dehydroepiandrosterone sulfate plus variable concentrations of the test compound.

Sterol 24-reductase

Sterol 24-reductase was assayed in preparations of rat liver microsomes by following the conversion of lanosterol to dihydrolanosterol by gas chromatography [31, 32]. Assays were conducted in 100 mM Tris, pH 7.5, containing 4 mM $MgCl_2$, 330 mM NADPH, 10 mM glutathione, 10 mM nicotinamide, 70 μM lanosterol, 0.15 M glucose, and 20 U of glutathione oxidase with 1.6 mg of microsomal protein. Inhibition studies with varying concentrations of epristeride were conducted in the presence of 70 μM lanosterol at saturating concentration of NADPH.

Inhibition studies with epristeride

All studies designed to determine the inhibitory potency of epristeride were conducted at constant concentrations of substrate near their experimentally determined K_m values. Inhibitor concentrations were varied to a maximum of 10 or 20 μM. With those systems that demonstrated significant inhibition, defined as >50% at 10 μM of the test compound, an apparent inhibition constant ($K_{i,app}$) was estimated by the method of Dixon [33]. Dead-end inhibition and multiple-inhibition studies of SR activities with the recombinant human isoenzymes were conducted essentially as described previously for the rat liver SR [9].

Data analysis

Data from Dixon analyses were computer fit to the equation for a linear function ($y = mx + b$) using the LINE program of the Fortran programs described by Cleland [25]. Data from dead-end inhibition studies were fit using the UNCOMP, NONCOMP, and COMP programs also as described [25]. Double inhibition experiments were analyzed by the same protocol as published [9].

Steroid receptor assays

The potential binding of epristeride to steroid receptors was determined by BioTex Associates (Lubbock, TX). The five systems with reference ligands were: (1) androgen receptor from hamster ventral prostate using [^{3}H]DHT and unlabeled DHT and T as reference compounds, (2) estrogen receptor from hamster uterus using [^{3}H]estradiol and unlabeled estradiol as the reference ligand, (3) progesterone receptor from estrogen-primed hamster uterus using [^{3}H]progesterone and unlabeled progesterone as the reference standard, (4) glucocorticoid receptor from rat liver with [^{3}H]dexamethasone and nonradioactive dexamethasone as the reference compound, and (5) mineralocorticoid receptor from hamster kidney using [^{3}H]aldosterone and unlabeled deoxycorticosterone as the reference standards.

Briefly, binding assays for each system involved the addition of a fixed amount of receptor preparation to a fixed amount of ^{3}H-steroidal standard (40,000 cpm) and a varying concentration of unlabeled competitor in a final set volume of 0.5 ml. Following an overnight incubation, the free steroids were removed by filtration under vacuum and the ^{3}H-steroid remaining bound to the receptor was determined. The relative binding activity (RBA) of each unlabeled steroid was determined from the concentration of competing ligand needed to reduce receptor binding by 50% (C_{50}) as compared to the reference compounds. The relative binding affinity of the test compound was calculated by dividing the C_{50} for the reference by the C_{50} of the test compound and multiplying by 100; the RBA of the reference compounds listed above thus are set at 100 for a baseline reference. Further details of the receptor binding protocols have been published by Leavitt *et al.* [34, 35].

RESULTS AND DISCUSSION

Selective inhibition of human SR type 2

The initial selection of epristeride as a potential clinical candidate [36] for treatment of DHT dependent prostate disease such as BPH was largely based on its potent inhibitory effects of the enzyme activity isolated from human prostatic tissue [7, 10]. The compound was also shown to be a potent inhibitor of the SR activities monitored in the rat prostate [37] and rat liver [9]. However, the different kinetic characteristics observed for the prostatic SR activities found between

species (rodent, dog, monkey, and human) [10] had led us to conclude that primates were the most relevant species in which to evaluate such uncompetitive inhibitors.

This conclusion has been strengthened with the subsequent recognition and identification of two SR forms in both rodent and man [11–14]. Interestingly, the primary amino acid homologies between the two isoenzymes within each species were only 45 to 50%. In addition, relatively low sequence homologies for each of the two SR isoenzymes were found between the two species: 60 and 75% for type 1 and 2, respectively. Functionally, the SR isoenzymes also differ significantly. Isoenzymes 1 and 2 from both species have the common characteristics of maximal catalytic activities at neutral and low pH, respectively. In addition, these isoenzymes diverge functionally in their sensitivities to representative 3-oxo-4-aza steroidal inhibitors. For example, 4MA is a potent inhibitor of both human and rat isoenzyme activities while finasteride, in contrast, is only a relatively weak inhibitor of the enzyme activity catalyzed by the human type 1 SR [11–14]. The SR isotypes of the two species also show differential tissue expression: both enzymes are present in the rat prostate at approximately equivalent levels of activites while isotype 2 clearly predominates in the prostate of man [13, 14]. It is likely that the SR activity we have monitored in the prostate of the dog, which is refractory to inhibition by epristeride ($K_{i,app} \sim 1000$ nM) in the pH range (6.6 to 7.0) of maximal tissue catalytic activity [10], also is an isoform 1 type of enzyme.

To ascertain the inhibitory effects of epristeride on the two human SR activities, both enzymes were expressed individually in a stable mammalian cell line. Our preliminary characterizations (not shown) of the pH profiles and steroidal substrate preferences for the two forms of human SR were consistent with data published by other investigators [11–14]. In addition, the reported differential inhibition of human isoenzymes 1 and 2 by 4MA and finasteride have been qualitatively confirmed as summarized in Table 1.

Table 1. Inhibition of SR isoenzymes types 1 and 2

Enzyme[a]	Epristeride $K_{i,app}$ (nM)[b]	Finasteride $K_{i,app}$ (nM)	4MA $K_{i,app}$ (nM)
Human			
Recombinant type 1	400–450	110–150	3
Recombinant type 2	0.7–2	1–3	2–3
Prostate (type 2)	2–15	4–20	4–10
Rat			
Prostate (type 1)	10–20	6	8–10
Liver (type 1)	5–10	5–6	4
Epididymus (type 2)	2–4	4	3

[a]SR activities were determined as described under Experimental. The SR isoenzymes monitored in tissue extracts were assigned by the pH of the individual assays: human prostate and rat epididymus, pH 5.0; rat prostate and rat liver, pH 7.0 or 7.5.

[b]Ranges for inhibitory potencies represent multiple determinations with different preparations of enzyme activity.

For reference, our inhibitory data for the two rat isoenzymes also are presented.

Using the two recombinant human SR activites, we were able to demonstrate that epristeride is a selective inhibitor of the human type 2 SR (Table 1). The selectivity of epristeride's inhibitory potency for type 2 is over 400-fold greater than that demonstrated on human isoenzyme 1. This differential inhibitory potency of epristeride in favor of human isoenzyme 2, the predominant activity found in the human prostate, appears greater than that measured for the competitive inhibitor finasteride which shows an approx. 50-fold preference for isoform 2 by this analysis. Collectively, both compounds appear to be potent dual inhibitors of the rat isoenzyme activities but are type 2 selective for the human enzymes. The data in Table 1 indicates that epristeride is a *more selective* inhibitor of the human type 2 SR activity than is finasteride.

The mechanism of SR inhibition with the steroidal acrylates has been shown to result from the formation of a complex with enzyme and $NADP^+$ using enzyme activity isolated from human prostate [7], rat prostate [37] and rat liver [9]. A consequence of this kinetic mechanism is the appearance of uncompetitive patterns versus the steroid substrate in double-reciprocal plots of dead-end inhibition data. Now, using the recombinant human enzymes described above, we have shown that epristeride is an uncompetitive inhibitor versus both substrates (T and NADPH) with each of the two human SR types (see data with T in Fig. 1). In contrast, finasteride has been shown to be a competitive inhibitor versus T with SR activities of the recombinant SR isoenzymes (data not shown; also see Refs [11–14]).

Upon further analysis employing double-inhibition experiments designed to test the synergistic association of $NADP^+$ and inhibitor to SR [9], it has been shown that the affinity of epristeride for both human SR isoenzymes is enhanced in the presence of this oxidized form of the cofactor ($\beta < 1$ in Table 2). These results indicate that the kinetic mechanisms of inhibition of the human SR isoforms 1 and 2 both derive from the formation of preferred ternary complexes consisting of epristeride, $NADP^+$, and the human enzyme isoform (for a more complete discussion of the kinetics associated with the inhibition of SR by the steroidal acrylates, see Ref. [9]).

Epristeride as an inhibitor of alternative enzyme activities

Since a lack of enzyme selectivity could presage further development, it was deemed important to discover whether a potential clinical candidate would have inhibitory activity on other enzymes having features of ligand binding and chemical catalysis that were common with that of SR. Our original observation that epristeride is a potent inhibitor of human prostatic SR activity, now recognized to be the type 2 isoenzyme, encouraged us to further characterize this molecule in assays for alternative activities prior to a complete preclinical safety evaluation leading to clinical studies

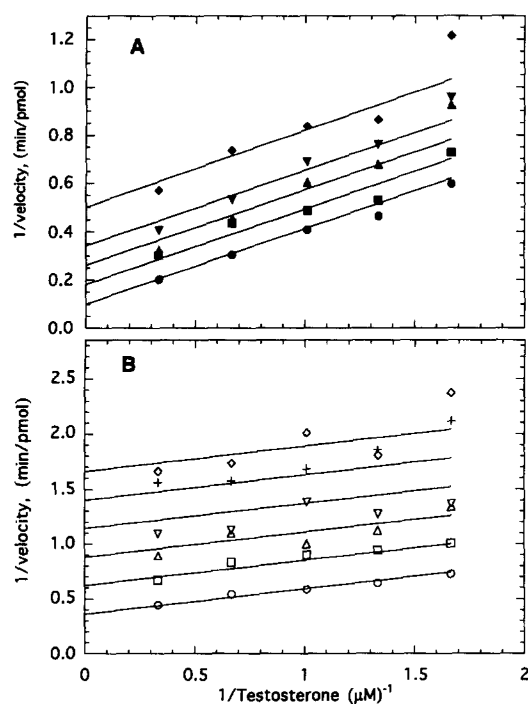

Fig. 1. Dead-end inhibition patterns of human recombinant SR types 1 and 2 in the presence of epristeride. Activities of human recombinant SR type 1 (A) and type 2 (B) were determined as described under Experimental. The concentrations of epristeride evaluated with isozyme 1 were 0 (●), 300 (■), 600 (▲), 900 (▼), and 1500 (◆) nM (A) and 0 (○), 0.1 (□), 0.2 (△), 0.3 (▽), 0.4 (+), and 0.5 (◇) nM with isozyme 2 as depicted in B. The data sets were evaluated with the UNCOMP program as described by Cleland [25]. Kinetic constants from these analyses are presented in Table 2.

in man. For this reason, several enzymes which catalyze chemical reactions different from, but related to, that involving SR were investigated as alternative targets for epristeride.

As an approach toward determining the specificity of this steroidal acrylate inhibitor for SR, seven alternative enzymes that function on steroidal substrates were selected. In considering the alternative enzyme systems to study which conceivably might interact with epristeride, two criteria were involved as primary determinants. First were systems whose catalytic mechanisms of action with steroidal substrates could lead to enzyme-bound "enol(ate)" intermediates. Here, it was felt that epristeride might show high-binding affinity for the alternative target by posing as a mimic of its high energy intermediate state—the same principle involved in the original design of the steroidal acrylates as inhibitors for SR [6–10]. Second were several ubiquitous enzyme systems that utilize steroidal substrates having structural features similar to ligands known to bind to SR; the ligands of interest were NADPH, T, DHT, the 3-oxo-4-aza steroids, such as finasteride and 4MA, and the steroidal acrylates as exemplified by epristeride.

Two enzyme activities whose inhibition could lead to a potential lack of specificity were identified through considerations of their mechanisms of action. Both processes, shown schematically in Fig. 2, involve probable enzyme intermediates having structural features similar to those incorporated into the steroidal acrylates as potent SR inhibitors. These characteristics include oxygen functionality at C-3 where $NAD(P)^+$ oxidation/reduction occurs and sp^2 hybridization at C-3 and C-4. The first of these, the conversion of 3β-hydroxy-5-ene-steroids to 3-keto-4-ene-steroids (scheme B, Fig. 2) involves two enzyme activities. The first activity, an NAD^+ dependent 3β-hydroxysteroid dehydrogenase, catalyzes formation of a 3-keto-5-ene-steroid which is followed by a 3-keto-5-ene-isomerase. Inhibition of this isomerization could block the biosynthesis of most steroid hormones ultimately leading to

Table 2. Inhibition constants of epristeride with recombinant human SR isoenzymes 1 and 2

Recombinant human enzyme	Variable substrate	V_{max} (pmol/min/mg)	Substrate K_m (μM)	Inhibition constant K_{ii} (nM)
		(*A*) *Dead-end inhibition analyses*[a]		
Type 1	Testosterone	34 ± 3	3.5 ± 0.5	350 ± 50
	NADPH	8 ± 1	12 ± 2	870 ± 60
Type 2	Testosterone	18 ± 2	0.3 ± 0.1	0.25 ± 0.05
	NADPH	20 ± 2	40 ± 5	0.30 ± 0.05
		(*B*) *Double inhibition analyses*[b]		
	Steroidal inhibitor	K_I (nM)	K_J (μM)	β
Type 1	Epristeride	250 ± 30	7 ± 1	0.7 ± 0.2
Type 2	Epristeride	0.7 ± 0.1	250 ± 50	0.5 ± 0.2

[a]All inhibition kinetic patterns with epristeride were best analyzed by the UNCOMP program described by Cleland [25]; results from alternative fits are not shown.

[b]Double-inhibition experiments were conducted as detailed previously for the rat liver SR activity [9] at pH 7.5 and 5.0 with the human recombinant types 1 and 2, respectively. The apparent dissociation constants of K_I and K_J are those for the two inhibitors epristeride and $NADP^+$, respectively. The value of β is an empirical value used to show the degree of cooperativity in binding of the two variable ligands; when $\beta < 1$, association of the two inhibitors to the enzyme is synergistic [9].

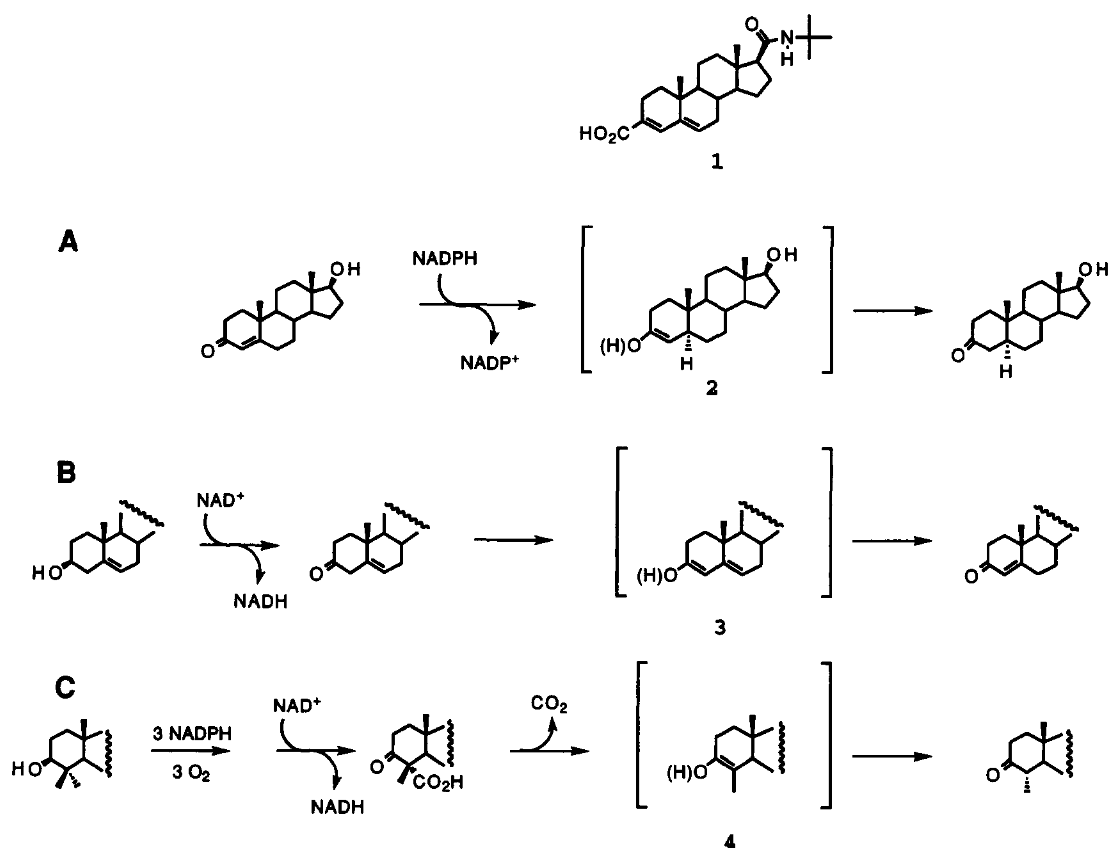

Fig. 2. Mechanisms of enzymes with steroidal enolate intermediates. Proposed enol(ate) intermediates in the chemical mechanisms for steroid 5α-reductase (2, scheme A), 3β-hydroxy-5-ene-steroid dehydrogenase/3-keto-5-ene-steroid isomerase (3, scheme B), and 3β-hydroxysteroid dehydrogenase/30α-decarboxylase (4, scheme C) are compared to the structure of epristeride (steroidal acrylate 1).

complications associated with complete androgen blockade.

The second enzymatic process of potential concern based on this set of criteria was the system which involves removal of the methyl groups at C-4 from lanosterol in the pathway to cholesterol biosynthesis (for a discussion of this pathway, see Ref. [38]). In the initial sequence of enzymatic events, the 4α-methyl group (C-30) is successively oxidized by a mixed function oxidase to the corresponding carboxylic acid. Each of the three sequential oxidations requires one equivalent of NADPH and of molecular oxygen. Subsequent dehydrogenation of the 3β-hydroxyl functionality to the ketone enables facile decarboxylation through an enzyme-bound 3,4-enolate intermediate. Protonation from the β-face of C-4, resulting in epimerization of the remaining methyl group, followed by reduction at C-3 to the alcohol allows for removal of the C-31 methyl group by the same enzymatic process.

The proposed enolate intermediates formed upon loss of C-30 and C-31 as CO_2 resemble the putative intermediate enolate in the mechanism of SR as shown in scheme C of Fig. 2. Thus, compounds targeted to block the conversion of T to DHT by inhibiting SR as intermediate state analogs also might inhibit 4-demethylation. Blocking the removal of C-30 and C-31 would be expected to result in the accumulation of 4,4-dimethyl sterols and prevent the biosynthesis of all steroids downstream in the pathway. The similarity of the proposed intermediate states for the 3β-hydroxy-5-ene-steroid dehydrogenase/3-keto-5-ene-steroid isomerase (structure 3) and 3β-hydroxysteroid dehydrogenase/30α-steroid decarboxylase (structure 4) to that for the SR catalyzed reaction (structure 2) are depicted in Fig. 2.

The coupled enzyme activities of 3β-hydroxy-5-ene-steroid dehydrogenase and 3-keto-5-ene-steroid isomerase, proposed to reside within the same polypeptide, were evaluated in a coupled reaction using pregnenolone as substrate. Minimal inhibition was observed at 1 μM of epristeride; further analysis gave an apparent inhibition constant ($K_{i,app}$) in excess of 2 μM. These results are in contrast to some of the 3-oxo-4-aza steroidal inhibitors of SR, such as 4MA, which are potent inhibitors of the 3β-dehydrogenase activity ($K_i < 10$ nM) [13]. It is interesting to note that the inhibition patterns of several of the 4-aza compounds have been determined to be uncompetitive versus 3-hydroxysteroid substrates, and competitive versus 3-ketosteroid substrates. These patterns are consistent with the formation of a tightly associated enzyme–NADPH-inhibitor complex being responsible for the potent inhibition of the this activity by the 3-oxo-4-aza steroids [22].

In order to assay the decarboxylation step involved in C-4 demethylation of sterols catalyzed by hydroxysteroid 3β-dehydrogenase/30α-decarboxylase, a novel substrate labeled with ^{14}C in the C-30 position ([30-^{14}C]cholestanol-4α-carboxylic acid) [24] was employed. This assay was designed in order to kinetically isolate the decarboxylation step involved in removal of these methyl groups. No inhibition of the decarboxylase activity was observed in the presence of epristeride at concentrations as high as 5 μM using this novel assay.

Additional enzyme systems were chosen upon consideration of the structural similarities between their known substrates or ligands and those for SR. Included in this selection were four enzymes: steroid 5β-reductase, which utilizes T and NADPH as substrates in a reaction that is mechanistically similar to that catalyzed by SR including an enolate intermediate; aromatase, which can utilize T and NADH as substrates; 3α-hydroxysteroid dehydrogenase, for which DHT can be a substrate, and steroid sulfatase, which utilizes substrates that possess anionic charge in the C-3 region of steroids as found in epristeride. Of additional concern was a key step in the biosynthesis of cholesterol from lanosterol involving reduction of the C-24(25) double bond. This process, catalyzed by sterol 24-reductase, is known to be inhibited by 24-aza sterols [31, 32]; consequently, the C-20(21) amide present in epristeride was of some consideration.

The results of these specificity studies are presented in Table 3. At a concentration of 5 μM, no inhibition of rat liver steroid 5β-reductase was observed, and only 20% inhibition was detected at 10 μM epristeride. Minimal inhibition was also determined for the 3α-hydroxysteroid dehydrogenase from rat liver where 64% of enzyme activity was maintained at 5 μM of epristeride. An apparent inhibition constant of greater than 4 μM was determined for epristeride with the 3α-hydroxysteroid dehydrogenase, an interaction that is comparable to the Michaelis constant we have determined ($K_m = 3$ μM) for androsterone as substrate.

Comparable results were obtained using the other three enzyme systems. With the steroid sulfatase from rat liver, an apparent inhibition constant of 2.5 μM was derived. Marginal inhibition (<40%) of sterol 24-reductase activity was seen initially at 10 μM inhibitor concentration; further analysis provided an estimate of the inhibitory potency as greater than or equal to 3 μM for the $K_{i,app}$. This inhibition was comparable to that observed for finasteride ($K_{i,app} \sim 3$ μM). Human placental aromatase maintained greater than 90% enzyme activity in the presence of 10 μM epristeride. Since several of the 3-oxo-4-aza steroids have been shown to be time-dependent inactivators of aromatase activity [29], an appropriate experiment to determine any similar behavior of the steroidal acrylate was undertaken. No time-dependent inhibition of human placental aromatase was observed under incubation conditions containing 10 μM epristeride and either NADPH or $NADP^+$.

The data discussed above, which is summarized in Table 3, establishes that epristeride is a potent inhibitor of only SR when compared to 7 alternative enzyme targets. Since the relatively high (micromolar) concentrations of epristeride that were necessary for observation of these inhibitory effects actually approach the limiting concentration of steroid that is soluble in the buffered assay solutions, the experimental $K_{i,app}$ values are thought to represent overestimates of the strengths of these interactions. In addition, these compounds exhibit a superior specificity-profile to some members of the 3-oxo-4-aza class of SR inhibitors which also interact with aromatase [29] and the 3β-hydroxysteroid dehydrogenase/5-ene-isomerase complex [22].

Interaction of epristeride with steroid hormone receptors

The 3-carboxylic acid of the steroidal acrylates represents a structural characteristic not found in mammalian steroid hormones. As such, it was considered likely that epristeride would demonstrate minimal, if any, interaction with steroid hormone receptors. Such

Table 3. Enzyme specificity of epristeride

Enzyme target	Inhibition in presence of epristeride
3β-Hydroxy-5-ene-steroid dehydrogenase/ 3-keto-5-ene-steroid isomerase	20% inhibition at 1000 nM; ($K_{i,app} > 2000$ nM)
3β-Hydroxysteroid dehydrogenase/ 30α-steroid decarboxylase	No inhibition at 5000 nM; ($K_{i,app} \gg 5000$ nM)
Steroid 5β-reductase	20% inhibition at 5000 nM; ($K_{i,app} > 10,000$ nM)
3α-Hydroxysteroid dehydrogenase	50–60% inhibition at 5000 nM; ($K_{i,app} = 4000$ nM)
Aromatase	<10% inhibition at 10,000 nM; ($K_{i,app} > 10,000$ nM)
Sterol 24-reductase	~40% inhibition 1 t 10,000 nM; ($K_{i,app} \geqslant 3000$ nM)
Steroid sulfatase	50% inhibition at 3000 nM; ($K_{i,app} = 2500$ nM)

Table 4. Evaluation of epristeride affinity for steroid hormone receptors

Hormone receptor	Reference ligand	Reference ligand C_{50} (M)[a]	Epristeride C_{50} (M)[a]	RBA[b] (%)
Androgen	DHT	3.7×10^{-9}	$>1.0 \times 10^{-5}$	<0.04
	T	5.4×10^{-9}	$>1.0 \times 10^{-5}$	<0.05
Estrogen	Estradiol	1.5×10^{-9}	$>1.0 \times 10^{-5}$	<0.02
Progesterone	Progesterone	2.7×10^{-9}	$>1.0 \times 10^{-5}$	<0.03
Glucocorticoid	Dexamethasone	9.0×10^{-8}	$>1.0 \times 10^{-5}$	<0.90
Mineralocorticoid	Deoxycorticosterone	2.7×10^{-9}	$>1.0 \times 10^{-5}$	<0.03

[a]C_{50} is the concentration of competitor needed to reduce the specific receptor ligand from binding to the receptor by 50%.

[b]RBA represents the relative binding affinity for epristeride as a percent of that of the reference ligand.

interactions with a potential clinical compound were considered undesirable since they would introduce secondary pharmacological effects unrelated to the blockade of DHT biosynthesis.

Consequently, epristeride was evaluated as a potential ligand for 5 steroid hormone receptors. Results of *in vitro* receptor binding assays with this compound are presented in Table 4. No significant binding was observed with any of the 5 receptors; the RBA estimated using the maximal C_{50} value of 10 μM determined by the upper limits of the concentration of test compound, for each receptor was <0.9% that of the reference ligand in each case. Upon inspection of the experimental data, the C_{50}, which is set to be >10 μM because of the poor displacement of reference ligands by epristeride, represents a conservative upper limit for binding of steroidal acrylate to the receptors. From this observation, the affinity demonstrated by epristeride for each of these 5 receptors is more than 1000-fold lower than its affinity for the human or rat prostatic SRs.

CONCLUSIONS

The data discussed above, summarized in Tables 1 to 4, establish that the high-affinity interaction of epristeride is specific for SR when compared to 7 alternative enzyme targets and 5 steroid receptors. Since the relatively high (micromolar) concentrations of epristeride that were necessary for observation of any effects on alternative targets actually approach the solubility of the steroidal acrylate, the experimental $K_{i,\mathrm{app}}$ and C_{50} values presented in Tables 3 and 4 represent conservative estimates for the strengths of these alternative interactions. From these considerations, the binding of this steroidal acrylate to alternative enzyme and receptor targets is estimated to be at least 10^3 times weaker than the *in vitro* inhibitory potencies of human and rat prostatic SRs.

The availability of both human SR isoenzymes in a recombinant expression system has enabled us to evaluate the relative inhibitory potencies of epristeride upon both human targets. As summarized in this manuscript, epristeride is a selective inhibitor of human SR type 2, the form which predominates in the prostate of man. Mechanistically, inhibition of both human isoenzymes results from the preferential formation of ternary complexes consisting of SR, epristeride and the oxidized form ($NADP^+$) of the enzyme's requisite cofactor.

Taken together, the summarized data indicate that epristeride is a specific and selective inhibitor of human SR type 2. Upon extrapolation of these *in vitro* analyses, steroidal acrylates such as epristeride would be expected to be effective in selectively lowering DHT levels in man. The difference in three orders of magnitude between binding of the steroidal acrylate to SR and alternative targets allows for an ample therapeutic window between inhibition of the desired enzyme and any undesired agonism or antagonism.

The results summarized in this manuscript are consistent with the independent observations that epristeride can antagonize T, but not DHT, induced prostatic growth in the castrated rat [39–41] and can specifically lower serum DHT concentrations in the rat [39, 40] and the nonhuman primate [41]. Preliminary evaluation of epristeride in man has shown a decrease in plasma DHT concentrations in a dose dependent manner upon oral administration [36]. With these results, the test for utility of compounds such as epristeride in treatment of DHT induced disorders must await their further ongoing clinical evaluation.

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